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The NF-κB signalling pathway in human diseases: from incontinentia pigmenti to ectodermal dysplasias and immune-deficiency syndromes

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The transcription factor NF-κB regulates the expression of numerous genes controlling the immune and stress responses, inflammatory reaction, cell adhesion, and protection against apoptosis. Incontinentia pigmenti (IP) is the first genetic disorder to be ascribed to NF-κB dysfunction. IP is an X-linked dominant genodermatosis antenatally lethal in males. A complex rearrangement of the *NEMO* (NF-κB essential modulator) gene accounts for 85% of IP patients, and results in undetectable NEMO protein and absent NF-κB activation. On the other hand, hypohidrotic/anhidrotic ectodermal dysplasia (HED/EDA) has been ascribed to at least three genes also involved in NF-κB activation: ectodysplasin (*EDA1*), EDA-receptor (*EDAR*) and EDAR-associated death domain (*EDARADD*). During hair follicle morphogenesis, EDAR is activated by ectodysplasin, and uses EDARADD as an adapter to build a signal transducing complex that leads to NF-κB activation. Hence, several forms of HED/EDA also result from impaired activation of the NF-κB cascade. Finally, hypomorphic *NEMO* mutations have been found to cause anhidrotic ectodermal dysplasia with immunodeficiency (EDA–ID), whilst stop codon mutations cause a more severe phenotype associating EDA–ID with osteopetrosis and lymphoedema (OL–EDA–ID). The immunological and infectious features observed in patients result from impaired NF-κB signalling, including cellular response to LPS, IL-1β, IL-18, TNF-α, Tlr2 and CD40 ligand. Consistently, mouse knockout models have shown the essential role of NF-κB in the immune, inflammatory and apoptotic responses. Unravelling the molecular bases of other forms of EDA not associated with mutations in *NEMO* will possibly implicate other components of the NF-κB signalling pathway.

THE NF-κB SIGNALLING CASCADE

The transcription factor NF-κB regulates the expression of genes controlling the immune and stress responses, inflammatory reaction, cell adhesion, and protection against apoptosis (for a review, see 1). NF-κB is composed of homo- or heterodimers of five proteins belonging to the Rel family. In the vast majority of cell types, NF-κB is kept inactive in the cytoplasm through association with inhibitory proteins of the IκB family: IκBα, IκBβ and IκBε. IκB molecules are phosphorylated on two critical serine residues in response to multiple stimuli such as cytokines, various stress signals, viral and bacterial infections. The most extensively studied signals are tumour necrosis factors (TNF), interleukin-1 (IL-1) and lipopolysaccharide (LPS). This modification allows its recognition by an ubiquitination complex, and, following polyubiquitination, IκBs are degraded by the proteasome machinery. As a consequence, free NF-κB enters the nucleus and activates transcription of its target genes (Fig. 1) (2).

For many years, the kinase phosphorylating IκB (IKK for IκB kinase) has remained elusive. Upon biochemical fractionation, it was eventually identified as a high-molecular-weight complex migrating around 700–900 kDa and containing two related catalytic subunits: IKKα/IKK1 and IKKβ/IKK2. IKK1 and IKK2 are highly homologous kinases, both containing an N-terminal kinase domain, a helix–loop–helix (HLH) and

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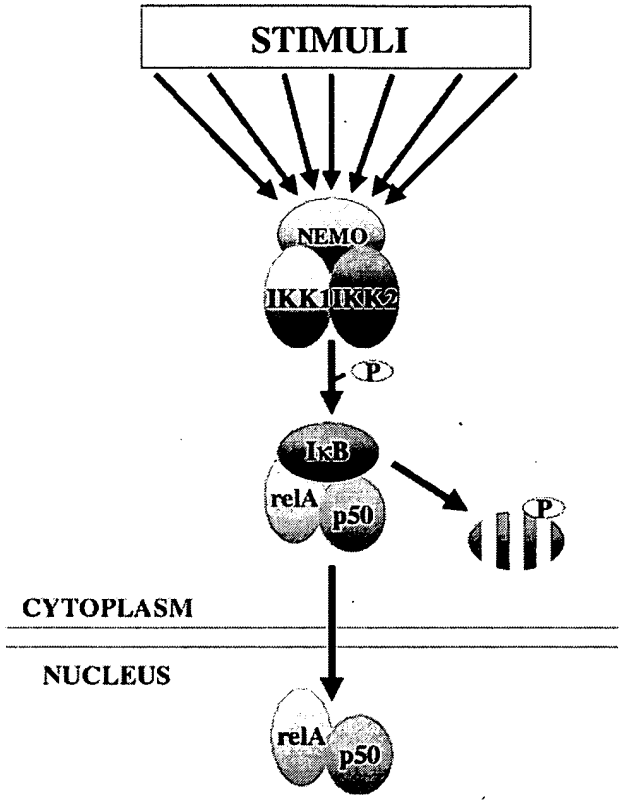

Figure 1. A simplified overview of the NF-κB activation process. In response to multiple stimuli, IKK kinase (composed of catalytic subunits IKK1 and IKK2 and regulatory subunit NEMO) is activated. IKK phosphorylates NF-κB inhibitory molecule (IκB), leading to its degradation by the proteasome and release of NF-κB dimer (the species composed of relA and p50 subunits is shown here). NF-κB translocates into the nucleus, where it regulates the expression of hundreds of genes. The NEMO subunit of IKK appears to be a convergence point, since its absence is associated with a lack of NF-κB activation in response to most known stimuli.

a leucine zipper domain (LZ) (for a review, see 2 and 3). Another of its components, NEMO (NF-κB essential modulator), also known as IKKγ, has been subsequently identified through various approaches (4,5). NEMO, in contrast to IKK1 and IKK2, exhibits no catalytic properties but acts as a structural and regulatory subunit of the IKK complex. Cell lines defective for NEMO do not activate NF-κB in response to most stimuli, demonstrating its essential role in the NF-κB activation process. In the absence of NEMO, a smaller complex (400 kDa) that contains both IKK1 and IKK2 could be found in mutant cell lines, but this complex was unresponsive to external stimuli. Structure prediction of NEMO indicates a high coiled-coil content and a C-terminal zinc finger. Deletion analysis has demonstrated that the N-terminal region of NEMO interacts with the C-terminal region of the IKK kinases, and that the C-terminal zinc finger is required for NF-κB activation by cytokines and LPS. The stoichiometry of the complex is currrently unknown.

Genetic models in which individual NF-κB or IκB proteins have been deleted in mice have confirmed the essential role played by NF-κB in the immune, inflammatory and apoptotic responses (6). Complete inhibition of NF-κB activity results in prenatal lethality due to TNF-induced liver apoptosis. Other knockouts (KOs) that alter but not abolish NF-κB activity all lead to multiple defects of the immune system affecting most cell lineages, often connected with abnormal control of the apoptotic response.

HUMAN DISEASES RELATED TO THE NF-κB PATHWAY

Incontinentia pigmenti

Incontinentia pigmenti (IP; MIM308300) is a rare X-linked dominant genodermatosis antenatally lethal in males. Affected females present with Blashko linear skin lesions occurring in four successive, sometimes overlapping, stages: erythema, vesicles, pustules (stage 1), verrucous lesions (stage 2), hyperpigmentation (stage 3), and pallor and scarring (stage 4) (7). Association with developmental anomalies of teeth, eyes, hair and the central nervous system have been reported. IP females show a completely skewed pattern of X inactivation, which results from selection of cells expressing the non-mutated X chromosome (8). The study of cells derived from spontaneously aborted fetuses revealed a complex rearrangement of the *NEMO* gene that results in the deletion of exons 4–10, potentially coding for a shortened ~130-amino-acid protein unable to elicit an NF-κB response. This recurrent rearrangement accounts for 85% of IP patients (9,10), and affected cells are indeed refractory to NF-κB-activating stimuli. Furthermore, IP cells are highly sensitive to TNF-induced apoptosis, suggesting a role for this cytokine in the development of IP lesions, and in associated anomalies as well (see below).

Ectodermal dysplasia

Ectodermal dysplasia (ED) is a clinically heterogeneous condition characterized by the abnormal development of ectoderm-derived structures, namely teeth, hair, nails and eccrine sweat glands (11). More than 170 phenotypes have hitherto been described. The hypohidrotic/anhidrotic form (HED/EDA) is characterized by the association of sparse hair, abnormal or missing teeth, and inability to sweat, which is responsible for life-threatening brain-damaging episodes due to hyperthermia (12). This clinically homogeneous phenotype has been ascribed to at least four genes, and three modes of inheritance have been reported: X-linked recessive (MIM 305100) and autosomal dominant and autosomal recessive (MIM 224900). The X-linked form is the most common, and is caused by mutations in the ectodysplasin gene (*EDA1*), a member of the TNF cytokine superfamily (13,14). Autosomal forms are caused by mutations in the *EDA3* gene (chromosome 2q13), which encodes EDAR, a protein belonging to the TNF-receptor superfamily (TNFR) (15,16). EDAR is the receptor of the EDA-A1 ectodysplasin isoform (17). This gene is responsible for both dominant and recessive HED/EDA. Recently, a mutation in the *EDARADD* gene (EDAR-associated death domain, chromosome 1q42) has been reported in another autosomal recessive form of HED/EDA (18). During hair

follicle morphogenesis and in the epidermis, EDAR is activated by EDA and uses EDARADD as an adapter to build an intracellular signal-transducing complex that leads to NF-κB activation (18,19). Biochemical analysis has shown that NF-κB activation by EDAR is NEMO-dependent (20). Not unexpectedly, patients carrying mutations in the *NEMO* gene also presented with EDA (20–22). EDA-A2, the second isoform of ectodysplasin, binds to XEDAR, a novel member of the TNF-R superfamily, which also activates NF-κB (23). Interestingly, a mutation in the *XEDAR* gene (X chromosome) has been recently identified in an HED/EDA patient (S.A. Wisniewski, B. Marszalek, K. Kobielak and W.H. Trzeciak, European Human Genetics Conference, May 2002). Mutations in the *EDA*, *EDAR*, *XEDAR* and *NEMO* genes reveal a new signal transduction pathway participating in differentiation of skin appendages.

Anhidrotic ectodermal dysplasia with immunodeficiency

A small number of male EDA patients have been reported with severe infections with pyogenic bacteria (e.g. *Streptococcus pneumoniae* and *Staphylococcus aureus*) and specific polysaccharide antibody deficiency (24,25), pointing towards a possibly distinct X-linked recessive entity causing EDA with impaired antibody response to polysaccharides (XL–EDA–ID). Following the recent report of a surviving male patient with a *NEMO* mutation (MIM 300248) (10), various hypomorphic *NEMO* mutations were found to cause distinct conditions. Mutations in the coding region are associated with the EDA–ID phenotype (MIM 300291), whilst stop codon mutations cause a clinically more severe syndrome associating osteopetrosis and/or lymphoedema with EDA–ID (OL–EDA–ID; MIM 300301) (9,10,20–22,26,27). Hence loss-of-function mutations cause IP, while hypomorphic mutations cause two allelic conditions, namely XL–OL–EDA–ID and EDA–ID.

EDA–ID males suffer from unusually severe life-threatening and recurrent bacterial infections of lower respiratory tract, skin, soft tissues, bones and gastrointestinal tract, as well as meningitis and septicemia in early childhood. The causative pathogens have most often been Gram-positive bacteria (*S. pneumoniae* and *S. aureus*), followed by Gram-negative bacteria (*Pseudomonas* spp. and *Haemophilus influenzae*) and mycobacteria. Interestingly, two children had *Pneumocystis carinii* infections (PCP), suggesting a more profound immunodeficiency. The two XL–OL–EDA–ID patients had a particularly severe ID, since they acquired environmental mycobacterial infections in their first year of life and died. As a comparison, severe combined immunodeficiency (lacking T cells) or interferon-γ (IFN-γ) receptor deficiency does not cause environmental mycobacteriosis so early in life.

Most patients have hypogammaglobulinaemia with low serum IgG (or IgG2) levels, while the levels of other immunoglobulin isotypes (IgA, IgM and IgE) varied. A number of EDA–ID patients have been described with elevated serum IgM levels (the 'hyper-IgM' phenotype) (20–22). Some, but not all, CD40-mediated signals are NEMO-dependent in B cells. In some patients, B cells have an impaired ability to switch in response to CD40 ligand (CD40L), and in others the switch is normal but there is an impaired proliferation and activation that also results in a 'hyper-IgM-like' phenotype. The impaired antibody response to polysaccharide antigens is the most consistent laboratory feature. When compared with these B-cell anomalies, patients with XL–EDA–ID and XL–OL–EDA–ID have normal T-cell proliferation to mitogens and antigens. Recently, impaired NK activity has been reported in some (28) but not all (20,27) patients with EDA–ID. The immunological abnormalities may be related to the type of *NEMO* mutation involved.

Thus, the immunological and infectious features of the patients result from an impaired cellular response of peripheral blood lymphocytes to LPS, IL-1β, IL-18, TNF-α, and CD40L (20). Other NF-κB-dependent pathways are likely to be affected as well. Indeed, NEMO-dependent NF-κB activation is important to the signalling pathways downstream of Toll receptors (Tlr) (29). These patients exhibit poor inflammatory response, also due to impaired cellular responses to pro-inflammatory cytokines (IL-1β, IL-18 and TNF-α) (20). Impaired response to LPS via Tlr4 and partially impaired CD40 signalling could explain the susceptibility of these patients to infections with Gram-negative bacteria and *P. carinii*, respectively. Infections by Gram-positive bacteria may result from impaired responses to IL-1β, IL-18, Tlr2 or other NF-κB-dependent signalling pathways. The occurrence of severe mycobacterial disease in these patients could be due to impaired IL-1β- and IL-18-dependent induction of IFN-γ, impaired cellular responses to IFN-γ-inducible TNF-α, or impaired signalling through Tlrs.

MOUSE MODELS OF NF-κB-RELATED DISEASES

NEMO-deficient mice have been produced in several laboratories. They are characterized by early lethality around embryonic day 12 in males (30–32). Death is due to massive liver apoptosis, a feature that has been previously observed in other KO models involving critical components of the NF-κB signalling pathway, such as relA or IKK2 (33–36). Whether lethality in male IP patients results from the same problem remains to be determined. Heterozygous NEMO-deficient female mice survive until birth, but, shortly after, exhibit a transient dermatosis, characterized by patchy skin lesions with massive granulocyte infiltration, hyperproliferation and increased apoptosis of keratinocytes (30–32). The whole process shares striking similarities with what is seen in IP patients. The only notable difference concerns the high level of morbidity that is observed in female mice, around postnatal day 6–10 (P6–P10), a feature that has never been observed in humans.

The recent generation of mice exhibiting a specific deletion of *IKK2* in the epidermis has also provided important information regarding the consequences of NF-κB dysfunction in skin, and may be relevant to IP dermatosis. Because *IKK2* KO mice die during early development, studying the postnatal role of IKK2 is presently impossible. A conditional *IKK2* KO in the epidermis has been produced first by targeting the *IKK2* locus with *loxP* sites then by crossing the mice with a strain expressing keratin 14 (K14)-driven Cre recombinase (37). Because K14 is specifically expressed in epidermis and hair follicles, these mice develop normally until P4–P5, when their skin starts to become hard and inflexible. This phenotype

progresses rapidly, and by P7–P8 the mice exhibit a highly rigid, shell-like skin with widespread scaling. At this stage, the mice become runted, and they subsequently die between P7 and P9. Histological analysis of skin sections at P7 shows epidermal thickening with loss of the granular layer, pronounced hyperkeratosis, focal parakeratosis, subcorneal pustule formation, increased cellularity and dilated blood vessels in the dermis. Interestingly, crossing the mice with TNFR KO mice suppresses these features, demonstrating the essential role of TNF in this process and suggesting that keratinocyte hyperproliferation is a secondary event resulting from inflammation.

A major difference between *NEMO* and *IKK2* skin KO mice is the high level of keratinocyte apoptosis observed in the former but not in the latter, thus representing a hallmark of IP. It is likely that the early stages of the disease in mouse models share many similarities with IP and deserve particular attention. Analysis of *IKK2* skin KO mice has demonstrated that NF-κB plays an essential role in controlling skin homeostasis, but the nature of the signal/change that triggers dermatosis remains unclear. Despite this uncertainty, the crucial role played by TNF in the development of the disease may suggest novel therapeutic approaches to treat the disease in IP and EDA–ID patients.

CONCLUSIONS

NF-κB dysfunction in humans appears to be associated with a broad range of defects involving many organs. This feature results from the central role played by this transcription factor in many signalling pathways critical for the immune, inflammatory and anti-apoptotic responses. The defects concerning the epidermis are of particular interest, since they confirm and extend the notion that NF-κB plays a key role in both homeostasis of the epidermis and development of skin appendages. IP is caused by lethal loss of NEMO function while XL–EDA–ID and XL–OL–EDA–ID are caused by hypomorphic *NEMO* mutations. EDA–ID is clinically heterogenous, since some patients have overwhelming clinical diseases caused by several microorganisms, whereas others seem to be susceptible to a limited number of species. Finally, most EDA elucidated to date involve ectodysplasin (EDA), its receptor (EDAR) or adaptor (EDARADD) in a signal-transducing complex that leads to NF-κB activation. Unravelling the molecular bases of other forms of EDA not associated with mutations in *NEMO* will possibly implicate other components of the NF-κB signaling pathway.

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